**Schistosoma mansoni** Excretory Circulating Cathodic Antigen Shares Lewis-x Epitopes With a Human Granulocyte Surface Antigen and Evokes Host Antibodies Mediating Complement-Dependent Lysis of Granulocytes

By Gouvert J. van Dam, Frans H.J. Claas, Maria Yazdanbakhsh, Yvonne C.M. Kruize, Antoinette C.I. van Keulen, Sonja T.M. Falcão Ferreira, J. Peter Rotmans, and André M. Deelder

Parasitic worms of the genus *Schistosoma* excrete relatively large amounts of immunogenic glycoproteins (circulating cathodic antigen [CCA]) that contain polysaccharide side chains with the trisaccharide Lewis-x (Le*) as a repeating unit. These carbohydrates evoke high titers of specific IgM antibodies that cross-react with the repeating Le* units on the surface of granulocytes. Consequently this might lead, in the presence of complement, to lysis of the granulocytes. In the present study, this hypothesis was investigated using anti--CCA mouse monoclonal antibodies (MoAbs) and polyclonal antibodies purified from sera of infected humans. By

SCHISTOSOMIASIS, a chronic disease that afflicts about 200 million individuals in the tropics, is caused by the infection with trematodes of the species *Schistosoma*. The schistosomes are blood–dwelling parasites, able to modulate and evade the host immune system in a variety of ways, such as camouflage by acquisition of host antigens, reduction of surface antigenicity by tegument antigen shedding, and eliciting parasite-protective blocking antibodies. In addition, most if not all pathology associated with the disease is immune-mediated, and therefore the study of immune-defense mechanisms in combination with induction of pathology is of major importance.

Recently, we reported the purification and structural analysis of one of the major antigens associated with the gut of *Schistosoma mansoni* worms, the circulating cathodic antigen (CCA). The antigenic moiety of CCA was characterized as a polysaccharide with the Lewis–x (Le*) trisaccharide as a repeating unit. Le* epitopes are found on glycolipids of a number of human cell types (such as granulocytes, cells of the urogenital tract, and adeno-carcinoma cells), as well as on free oligosaccharides in human milk and urine. Circulating human granulocytes are enriched in Le* and carry in relatively high abundance branched N–linked polysaccharides that have Le* repeating units. Suggestive evidence was provided by Deelder et al that the predominant IgM response against *S. mansoni* gut–associated antigens in humans was directed against CCA.

In chronic schistosomiasis patients, a mild to moderate neutropenia has been reported and has been attributed to a possible inhibitory factor in the sera of these patients delaying the maturation of neutrophils in the bone marrow and spleen. Combining these observations we proposed the hypothesis that excretion of CCA evokes high titers of anti–poly–Le* antibodies, which are also directed against identical host carbohydrate structures such as found on neutrophils, thereby causing complement mediated Ab-dependent lysis of these cells. In this context, Ko et al found that a murine protective IgM MoAb, raised against *S. mansoni* eggs, recognized the Le* determinant (also called stage–specific embryonic antigen–1 [SSEA–1]), and showed binding to the surface of live schistosomula. Recently, it has been shown that sera from *Schistosoma*–infected individuals displayed reactivity against Le* antigens on the surface of HL60 cell lines, mediating complement–dependent lysis of these cells.

In this study, we have indeed shown that anti–CCA antibodies can be involved in granulocyte lysis. By demonstrating that MoAbs directed against CCA recognize purified human granulocytes, in which the presence of complement, cause lysis of these cells; (2) demonstrating that anti–CCA IgM antibody levels in sera from schistosomiasis patients correlated significantly with complement–dependent lysis of purified human granulocytes; and finally (3) showing that anti–CCA IgM antibodies immunopurified from infected human sera caused lysis of granulocytes in a complement–dependent cytotoxicity assay.

MATERIALS AND METHODS

Production and specificity of MoAbs. MoAbs were used from different cell lines obtained from various fusions using immunized mice or mice infected with *S. mansoni*, *S. haematobium*, or *Schisto soma japonicum*. Specificity was tested using immunofluorescence and immunoelectrophoresis. MoAbs were selected that showed reactivity with CCA or another schistosome gut–associated antigen, circulating anodic antigen (CAA) as a control. The primary structure of CAA has also recently been characterized and has been shown to be completely different from the structure of CCA, wherefore anti–CAA MoAbs were taken as control MoAbs. Human serum samples. Thirty–nine sera with anti–CCA antibodies were selected for the present study, 29 of which (group I) were used in previous studies on anti–CCA reactivity, while 10 sera (group II) were randomly taken from sera that showed positive

From www.bloodjournal.org by guest on August 16, 2017. For personal use only.
reaction in an immunofluorescence assay on sections of Rossman's fixed adult Schistosoma worms. The sera in group I were from patients whose infections were parasitologically proven by demonstration of S mansoni eggs in the stool. As negative controls, eight sera with a negative immunofluorescence reaction were used (group III). Ten sera (group IV) of persons with helminth infections other than Schistosoma, exhibiting high antibody titers against Fasciola hepatica (n = 1), Onchocerca volvulus (n = 4), Echinococcus granulosus (n = 1), Loa loa (n = 2), or Strongyloides stercoralis (n = 2) were also tested.

Antibody detection assays. Patient sera were tested in an immunofluorescence assay, as well as in an Ab-capture enzyme-linked immunosorbent assay (ELISA) for anti-CCA IgM antibodies. The ELISA for anti-CCA IgG antibodies was performed similarly as described for the IgM assay, with rabbit antihuman IgG (Dakopatts, Glostrup, Denmark) as coating antibodies. The cut-off levels for the ELISAs were determined on the basis of the negative controls (group III, average activity ± 2 SD). As only relative units were relevant in this study, results are expressed relative to the serum with the highest reactivity.

Binding of MoAbs to granulocytes using flow cytometry. Granulocytes from healthy donors were isolated on Ficoll density gradient centrifugation. After a 30-minute incubation on ice with 100 μg MoAb/mL buffer (1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS], pH 7.8), cells were washed and incubated for 30 minutes in the dark with 40 μL fluorescein isothiocyanate (FITC)-conjugated goat antimouse Ig antibodies (1/25 diluted; Becton Dickinson, San Jose, CA). After washing, the sera remained overnight in 1% BSA in PBS at 4°C and the samples were run on a FACStar flow cytometer (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm, 300 mW. Ten thousand cells were measured per sample.

Complement-dependent lysis of granulocytes. Granulocytes were isolated from EDTA-treated blood of two healthy donors, labeled with carboxyfluorescein diacetate, and washed. After incubation of 1 μL of cells (4 × 10⁶/mL) and 1 μL heat–inactivated serum or MoAb solution for 60 minutes at 20°C and centrifugation for 5 minutes at 150g, the wells were flooded with RPMI and the excess fluid gently aspirated. Next, 5 μL of rabbit serum was added as a source of complement (as is commonly used in serologic cross-matches in transplantation). After incubation for another 90 minutes at 20°C, the complement reaction was stopped by addition of 1 μL 2.5% EDTA, which contained 0.03% ethidium bromide to label the nuclei of the dead granulocytes. Cytotoxicity was scored using a fluorescent microscope equipped with an excitation and barrier filter to allow simultaneous visualization of green and red fluorescence. To discriminate between IgM- and IgG-mediated lysis, sera were also treated with dithiothreitol (DTT), which specifically degrades IgM antibodies. The score of the granulocytotoxic activity, expressed on a scale of 0 to 5 (corresponding, respectively, to 0% and 100% lysis of the cells), was averaged for the two donors and samples with scores greater than 0.5 were taken as positive.

ELISA on crude granulocyte antigen. Granulocytes (2 × 10⁶ in 0.6 mL PBS) isolated from normal human donors (see earlier) were disrupted by several cycles of freezing and thawing. The sample was divided in two, and half was treated with trichloroacetic acid (TCA); final concentration, 7.5%, which is the usual concentration for isolation of a CCA–enriched S mansoni worm antigen preparation (TCA–soluble adult worm antigen [AWA–TCA]) and neutralized by 4 mol/L NaOH. To the other half 50% Tween–20 in PBS was added to a final concentration of 0.3%. Both of these preparations were centrifuged and diluted in a dilution series in the CCA-capture ELISA. Absorbances were read against a standard curve of AWA–TCA, which contains 3% CCA, and the sample concentrations were expressed in nanograms CCA (or nanograms CCA-equivalent reactivity).

Purification of anti-CCA antibodies from human sera. Immuno-purified CCA was coupled to Reacti-Gel (6X; Pierce, Rockford, ME), using the standard procedure supplied by the manufacturer. Fifty-microliter samples of serum from infected humans were mixed with 100 μL (in PBS) of coupled gel, and incubated for 1 hour at 8°C. After washing four times with PBS, the antibodies were dissociated from the gel by incubation with 200 μL of a thiocyanate buffer (3 mol/L NaSCN, pH 6.2), for 30 minutes at 4°C. The eluted antibodies were washed seven times with PBS using Ultrafree-MC filters (type PLTK; 30-Kd limit; Millipore, Bedford, MA) and finally diluted in 100 μL PBS. These sera were tested in the ELISA for anti-CCA IgM antibodies as described earlier. As a negative control, the sera were also incubated with BSA–coupled gel and eluted by sodium thiocyanate (NaSCN), using the same procedure as described earlier.

RESULTS

Binding of MoAbs to granulocytes and lysis. Of 21 anti-CCA MoAbs tested at 100 or 10 μg/mL, 17 clearly reacted with granulocytes and three were marginally positive, while one MoAb showed no binding (representative data shown in Fig 1). Four control MoAbs were negative. On the basis of these results, we also tested whether IgM antibodies in sera from schistosomiasis patients bound to granulocytes in a similar way. However, no binding of IgM antibodies from infected human sera could be detected by flow cytometry.

Three IgM and one IgG3 anti-CCA MoAbs were tested for granulocytotoxicity and found to be highly granulocytolytic, while two anti-CAA MoAbs (IgM and IgG3) were negative at the same concentrations used (0.1 mg/mL). To evaluate at which antibody concentration MoAbs still exhib-
caused by IgM and IgG serum antibodies was shown for retained their CCA-binding activity as determined in ELISA.

...tions up to 0.5 mg/mL, where the native antibodies were anti-CCA antibodies, all sera in group I and endemic control individuals or individuals with negative schistosomiasis serology or from nonendemic control individuals (group III), or from individuals with other parasitic infections (group IV). Horizontal bars represent the means of the different groups.

Anti-CCA antibodies in sera and lysis of granulocytes. We have examined four well-defined groups of patients: group I included patients with parasitologically confirmed schistosomiasis; group II consisted of individuals with only serologically proven schistosomiasis; group III included nonendemic control individuals or individuals with negative schistosomiasis serology; and group IV consisted of individuals with other parasitic infections. In the ELISA for IgM anti-CCA antibodies, all sera in group I and II were positive (Fig 2A). IgG anti-CCA antibodies were found in 83% of the sera from patients in group I and 50% in group II, respectively. One of the negative control sera in group I and 50% of group II (Fig 2B). Significant statistical differences for IgM anti-CCA antibody levels were found between group I and III and between group II and III.

Significant complement-dependent lysis of granulocytes caused by IgM and IgG serum antibodies was shown for 83% of the sera from patients in group I and 50% in group II, respectively. One of the negative control sera in group III was positive, while all sera of individuals with parasitic infections other than Schistosoma were negative (Fig 3A). After treatment with DTT, only 34% of the sera in group I remained positive, whereas all sera in groups II to IV were negative (Fig 3B), which indicates that most cytolytic reactivity resulted from serum antibodies of the IgM class.

A significant correlation was found between the IgM anti-CCA reactivity and the IgM-mediated granulocyte lysis in the sera from the patients of group I, II, and III (r = 0.37, P = 0.01, n = 47), while this was not the case for the IgG antibodies (r = -0.02, P = 0.9, n = 46). Granulocytotoxicity was indeed complement-dependent, as was shown by the complete abrogation after complement inactivation of the reactivity of two selected sera that were positive using untreated complement. Three sera of patients with non-Schistosoma helminth infections (O volvulus, E granulosus, L loa) showed low to moderate anti-CCA reactivity in the IgM-ELISA (Fig 2A), but granulocyte lysis was not observed for any of the 10 sera (Fig 3).

Presence of CCA-reactivity in crude granulocyte antigen. After TCA treatment of lysed granulocytes, no reactivity was observed in the CCA ELISA. However, treatment with 0.3% Tween-20 produced an antigen preparation that was clearly positive, yielding approximately 40 ng CCA-equivalent reactivity per 10^6 granulocytes.

Reactivities of immunopurified anti-CCA antibodies from human sera. Human anti-CCA antibodies immunopurified from sera of Schistosoma-infected individuals on immobilized CCA, showed a clear granulocytotoxicity (Table 1). No anti-CCA antibody reactivity could be isolated using an identical procedure with immobilized BSA.

DISCUSSION

Although schistosome gut-associated antigens CAA and CCA have been extensively studied over the years by several groups, an experimentally supported function or role of these antigens has not yet been given. Recent purification and structural analysis of both antigens presented new data and tools to extend the previous studies. As a result, it was described that CAA forms complexes with C1q and possibly interferes with the C1q-C1q-receptor interaction. Moreover, CCA was shown to be structurally similar
SCHISTOSOMA CCA INDUCES GRANULOCYTOTOXIC ANTIBODIES

Fig 3. Lysis of granulocytes by sera from individuals with parasitologically confirmed schistosomiasis (group I), with only serologically proven schistosomiasis (group II), with negative schistosomiasis serology or from nonendemic control individuals (group III), or from individuals with other parasitic infections (group IV). (A) Untreated sera (lysis by IgM and IgG antibodies; (B) DTT-treated sera (IgM antibodies are destroyed). Horizontal bars represent the means of the different groups.

to a major granulocyte surface antigen, and as high anti-
CCA IgM antibody titers are found in schistosomiasis pa-
tient sera combined with the mild to moderate neutropenia
observed in these patients, the hypothesis that CCA is in-
volved in a parasite-induced neutropenia was tested. In the
present report, we showed evidence to support this hypothe-
sis by first demonstrating that anti-CCA MoAbs bound to
an antigen (or antigens) present at the surface of granulo-
cytes, which in the presence of complement caused cell lysis.
Additionally, we presented evidence that schistosomiasis pa-
tient sera contained antibodies (predominantly IgM) that rec-
ognized granulocytes and induced in vitro complement-me-
diated lysis of the cells. Purification of these antibodies using
immobilized CCA proved that the granulocytolytic antibod-
ies are directed against CCA.

The demonstration that a crude granulocyte antigen prep-
paration reacts positively in the antigen-capture CCA ELISA
further confirmed that granulocytes contained antigenic
structures with repeating Le" epitopes as recognized by anti-
CCA antibodies. None of the 10 sera from patients with
helminthic infections other than Schistosoma showed sig-
nificant granulocytolytic activity, which supports the para-
site-specificity of the phenomenon.

Ko et al found that a mouse MoAb that was developed
against Schistosoma egg antigens and showed in vitro Ab-
dependent cellular killing of schistosomula, recognized the
SSEA-1 determinant (Le"). These investigators suggested
that patient antibodies directed against Le" might be a com-
ponent of concomitant immunity. However, in our hands,
infusion of three anti-CCA MoAbs before and during the
first days of a S mansoni infection in mice did not result in
any reduction of worm burden (unpublished observations:
also suggested the expression of autoimmunity following the
induction of anti-Le" antibodies, as these antibodies could
be directed against circulating granulocytes. Recently, it
has been shown that schistosomiasis patient sera contained
antibodies against Le" antigens present on HL60 cell lines.
In the present study, we have shown that antibodies that are
directed against the excretory antigen CCA, are involved in
complement-mediated lysis of purified human granulocytes.
Since a number of the anti-CCA MoAbs recognize egg
antigens, it is also possible that antibodies induced by egg
antibodies achieve a similar effect.

It has been described that in mice infected with S mansoni,
the general neutrophil inflammatory response was decreased,
caused by a depletion of mature neutrophils. This study
concluded that this decrease is due to a delay in the matura-
tion of the neutrophils in the bone marrow, but the present
study shows that it might also be caused by a granulocyto-
lytic process involving anti-Le" antibodies and mature gran-
ulocytes expressing multiple Le" epitopes. Another study
showed a decreased chemotactic response of granulocytes
in Schistosoma-infected mice as compared with uninfected
mice. Besides the lytic effect on the cells, the anti-CCA

Table 1. Reactivity of Native Sera and Immunopurified Human
Anti-CCA Antibodies in ELISA and Granulocytotoxicity Assay

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Anti-CCA IgM ELISA</th>
<th>Granulocytotoxicity Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Immunopurified</td>
</tr>
<tr>
<td>1</td>
<td>0.39</td>
<td>-0.01</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.55</td>
<td>0.34</td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>0.12</td>
</tr>
</tbody>
</table>

ELISA results are expressed relatively to the serum with the highest
reactivity, and granulocytotoxicity is scored on a scale of 0 to 5.
antibodies might also inhibit certain granulocyte functions like transmigration of adhesion mechanisms.

In addition to an effect on granulocytes through the induction of anti-Le^A antibodies, CCA might have a direct anti-inflammatory and antithrombogenic effect by acting as an inhibitor for the adherence of neutrophils to P-selectin on endothelial cells.\textsuperscript{45,46} CCA is detected in the circulation in concentrations sometimes as high as 1 μg/mL, but as an excretory antigen it might locally be present around the parasite in much higher concentrations.

In the present report we have shown, for the first time, that antibodies induced by a major schistosome antigen are involved in specific lysis of host granulocytes. This confirms the hypothesis that excretion of CCA and subsequent induction of high titers of anti-CCA antibodies might be one of the mechanisms the schistosomes use to ineflectuate the host-defense system. As a probable result of the anti-CCA antibody-induced neutropenia, schistosomiasis patients show a sensitivity for chronic microbial infections,\textsuperscript{47-50} but an increased susceptibility of these patients for bacterial infections as compared with patients having other parasitic infections has yet to be determined. Further studies will involve the association of levels of anti-CCA antibodies with neutrophil counts, as well as granulocyte function.

Although several functions of CCA have been suggested, this is the first experimentally supported role of CCA in the host–parasite interaction. It must be noted that the primary function of the synthesis of this antigen by the parasite, supported by the mucin–like nature of the antigen,\textsuperscript{10,14} might still be a direct physiologic or mechanical function, like protection of the parasite gut. The granulocytolytic role as described here may have evolved in the host–parasite relationship as an additional function for this molecule.

ACKNOWLEDGMENT

We gratefully acknowledge the technical assistance of Marian Witvliet (granulocytotoxicity experiments) and Maarten van der Keur (flow cytometry). We thank Jaco Verwey for providing the sera of patients with parasitic infections.

REFERENCES

5. Pearce EJ, Basch PF, Sher A: Evidence that the reduced surface antigenicity of developing Schistosoma mansoni is due to antigen shedding rather than host molecule acquisition. Parasite Immunol 8:79, 1986
16. Öntöfö TF: Carbohydrate changes in bladder carcinomas. APMIS 100:181, 1992
23. Ko AI, Dräger UC, Harn DA: A Schistosoma mansoni epito
recognized by a protective monoclonal antibody is identical to the stage-specific embryonic antigen 1. Proc Natl Acad Sci USA 87:4159, 1990


Schistosoma mansoni excretory circulating cathodic antigen shares Lewis-\textsuperscript{x} epitopes with a human granulocyte surface antigen and evokes host antibodies mediating complement-dependent lysis of granulocytes

GJ van Dam, FH Claas, M Yazdanbakhsh, YC Kruize, AC van Keulen, ST Ferreira, JP Rotmans and AM Deelder